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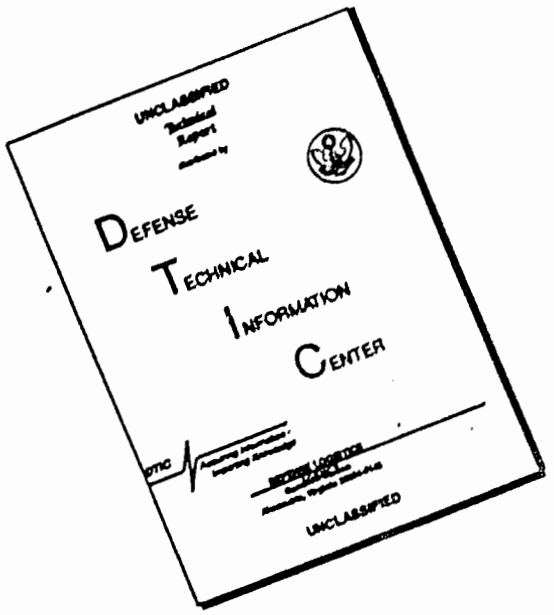

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APPENDICES

(1)

BASAL GANGLIA DOPAMINE-γ-AMINOBUTYRIC ACID-ACETYLCHOLINE INTERACTION IN ORGANOPHOSPHATE-INDUCED NEUROTOXICITY

FIRST ANNUAL REPORT

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APPENDIX 1-A

VARIATION OF COMMERCIAL DIISOPROPYL FLUOROPHOSPHATE PREPARATIONS IN TOXICOLOGICAL STUDIES

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ABSTRACT

The variation of three diisopropyl fluorophosphate (DFP) lots obtained from two sources was investigated. The effects of different lots of DFP on body weight, mortality, and brain acetylcholinesterase activity in rats varied significantly. The in vitro effects of these DFP preparations on brain acetylcholinesterase activity also were markedly different. It appears that different commercial preparations of DFP possess different potencies and that the same apparent doses of different DFP preparations will not produce the same effects upon any physiological system under study.

INTRODUCTION

Diisopropyl fluorophosphate (DFP) is an odorless, colorless volatile liquid[1] which is commonly used in research laboratories as an irreversible cholinesterase inhibitor[2,3]. Although DFP is very toxic to all species of animals, there is difference in susceptibility among species[4]. In an attempt to initiate the studies on neurotoxicity induced by DFP, we have encountered difficulty due to variations in potency when different lots of DFP were tested. Therefore, the present communication reports on experiments designed to study the variation of three DFP lots obtained from two sources. The study includes the effects of different lots of DFP on body weight and brain acetylcholinesterase activity in rats. The in vitro effects of these DFP preparations on brain acetylcholinesterase activities also are compared.

MATERIALS AND METHODS

Materials and animals: The three different diisopropyl fluorophosphate (DFP) preparations used in this experiment were purchased from Calbiochem-Behring Corp. (La Jolla, CA) and Aldrich Chemical Co. (Milwaukee, WI). They are designated as DFP-1 (Lot 101318, Calbiochem), DFP-2 (Lot 103288, Calbiochem) and DFP-3 (Lot 1029PH, Aldrich). Each preparation was sealed in a glass vial with 1 gram in each vial. Each 1 gram unit of DFP was diluted with saline to make up 100 ml. This stock solution was then divided into 5 to 10 ml aliquots in test tubes. They were then stored in a freezer. Different lots of DFP were prepared at the same time. The desired concentrations of DFP were freshly prepared before use. Acetylthiocholine iodide and 5:5-dithio-bis-2-nitro-benzoic acid (DTNB) also were obtained from Calbiochem (La Jolla, CA). Male Sprague-Dawley rats weighting 175 to 200 grams from Charles River Breeding Labs (Wilmington, MA) were used throughout the study.

Treatments of animals: In the lethality study, rats were administered with various doses of DFP ranging from 1 to 8 mg/kg, s.c., depending upon the different preparations. Mortalities occurring within 24 hours after the administration of DFP were recorded. The number of animals which died at a certain DFP dose during various experiments are summarized in Table 1.

When the potencies of different DFP preparations were compared, 4 groups of rats with 3 rats in each group received saline (1 ml/kg), DFP-1, DFP-2 and DFP-3, 2 mg/kg, s.c., respectively. The body weight of each rat prior to and 24 hours after the administration of saline or DFP was recorded. All groups of rats were sacrificed by decapitation 24 hours after the treatment. Whole brain acetylcholinesterase activity of each animal was assayed.

In another experiment, the *in vitro* effects of the different DFP preparations on acetylcholinesterase activity in rat brain homogenate also were determined. Dose-response effects for each

DFP preparation were studied with at least four DFP concentrations.

Biochemical assay for acetylcholinesterase activity: The colorimetric method of acetylcholinesterase activity by Ellman et al.[5] was used. Each brain was homogenized with 0.1M phosphate buffer, pH 8.0, in a Potter-Elvehjem homogenizer with 20 mg of tissue per ml. Two 0.4 ml aliquots of this homogenate were added to separate cuvettes containing 2.6 ml of 0.1M phosphate buffer, pH 8.0. DTNB reagent (0.01M in 0.1M phosphate buffer, pH 7.0), 100 µl, were then added to each cuvette. Twenty µl acetylthiocholine iodide (0.075M) were then added to the sample cuvette. The absorbance was measured at 412 mµ with a Beckman-2600K spectrophotometer and changes in absorbance were recorded. In studies of the in vitro inhibitory effects of DFP on brain acetylcholinesterase activity, the same procedures were used except different concentrations of DFP were added directly to the reaction mixture.

RESULTS and DISCUSSION

Effects of different DFP preparations on lethality in rats: There were significant differences in potency on mortality rate in rats treated with different DFP preparations. As shown in Table 1, DFP-2 was the most potent preparation among the three tested.

TABLE 1

Effects of Different DFP Preparations on Lethality in Rats

DOSE	MORTALITY*		
mg/kg, s.c.	DFP-1	DFP-2	DFP-3
1 mg/kg	0/10	0/16	
2 mg/kg	0/20	3/30	0/3
4 mg/kg	0/20	8/8	0/3
5 mg/kg	0/10	4/4	
6 mg/kg	8/8		
8 mg/kg	4/4		

*Number of animals that died / Total number of animals tested at a dose

Effects of a single administration of different DFP preparations on body weight and brain acetylcholinesterase activity in rats: The body weight and brain acetylcholinesterase activity in rats treated with the same dose (2 mg/kg, s.c.), of the three DFP preparations also were compared. As shown in Table 2, body weights of rats treated with DFP-1 were not significantly different from those of the saline-treated controls 24 hours after DFP was injected. In contrast, both DFP-2 and DFP-3 treated rats lost 11% of the body weight as compared with a 4% weight gain in control animals. Thus, DFP-1 was less potent than the other two preparations.

As far as the acetylcholinesterase activity in brain is concerned, the different batches of DFP also exhibited marked variations in the degree of enzyme inhibition. As also shown in Table 2, the brain acetylcholinesterase activities were 45.3, 64.3 and 53.6% inhibited in DFP-1, DFP-2 and DFP-3 treated animals, respectively. These differences in brain acetylcholinesterase inhibition by the three DFP preparations were statistically significant. Thus the order of potency was DFP-2>DFP-3>DFP-1.

The in vitro effects of different DFP preparations on brain acetylcholinesterase activity: Different DFP preparations also exhibited significant differences in the degree of inhibition of brain acetylcholinesterase activity when they were tested in vitro. As shown in Figure 1A, all three preparations exhibited concentration-dependent inhibition of brain acetylcholinesterase activity. The median inhibitory concentrations (IC50s) were 18, 12 and 14 µg/ml for DFP-1, DFP-2 and DFP-3, respectively. When these IC_{50} were compared with the brain acetylcholinesterase activity in animals treated with DFP, 2 mg/kg, s.c., for 24 hours, there was good correlation between in vitro and in vivo data (Figure 1B).

The results of these experiments have demonstrated the necessity for knowing (and reporting) acetylcholinesterase inhibitory potency of any preparation of DFP to be used in research

TABLE 2

Effects of Single Administration of Different DFP Preparations on Body Weights and Brain Acetyocholinesterase Activity in Rats

Treatments 2 mg/kg, s.c.	Body Weight, g ± S.E. Before Administration	24 Hrs after Administration	Brain Acetylcholinesterase Activity μmoles of acetylthiocholine iodide ± S.E. min/gram
Saline	185.3 ± 7.3	192.7 ± 5.5	7.76 ± 0.20
DFP-1	179.3 ± 0.6	182.7 ± 3.8	4.20 ± 0.11*
DFP-2	183.3 ± 4.4	163.3 ± 4.3*+	2.77 ± 0.18*+‡
DFP-3	184.7 ± 8.8	162.7 ± 6.2*+	3.60 ± 0.10*+

DFP-1: Lot 101318, Calbiochem

DFP-2: Lot 103288, Calbiochem

DFP-3: Lot 1029PH, Aldrich

Symbols *, + and ‡ are the indications of significance ($p < 0.05$)

*: All DFP groups compared with the control group

+: DFP-2 or DFP-3 compared with DFP-1

‡ : DFP-2 compared with DFP-3

investigations. Since it is apparent that different commercial preparations of DFP do possess different potencies, we can expect that the same mg/kg doses of different preparations will not produce the same effects upon any physiological system under study. We therefore suggest, on the basis of the present report, that investigators determine and report the IC_{50} of any DFP preparation being studied, and further report doses used as fractions or multiples of the IC_{50} value.

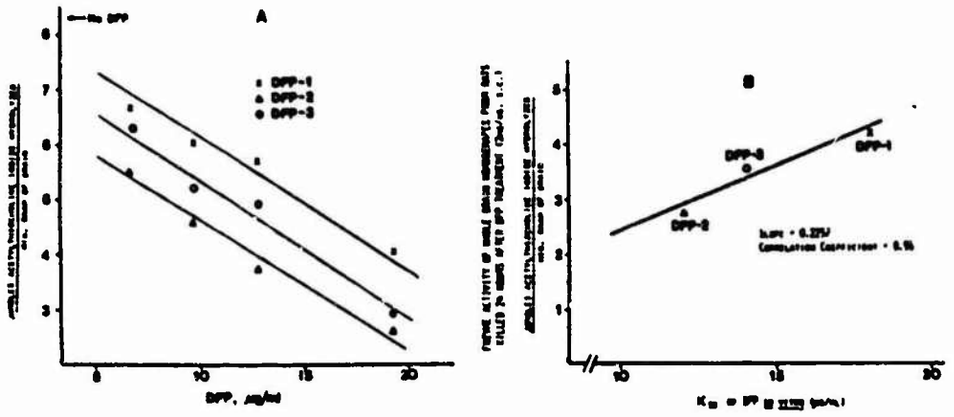

Figure 1. A. In vitro effects of different DFP preparations on rat brain acetylcholinesterase activities. Four different concentrations of DFP were tested in duplicate. B. Correlation between in vivo and in vitro effects of different preparations of DFP on rat brain acetylcholinesterase activity.

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APPENDIX 1-B

ASSESSMENT OF DIISOPROPYLFLUOROPHOSPHATE (DFP) TOXICITY AND TOLERANCE IN RATS

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ABSTRACT

Single s.c. injection of 1, 2, 3 or 4 mg/kg of DFP in saline was given to male rats. Control rats received saline. Beginning 24 hr after injections, body weights and food and water consumption were measured at 24 hr intervals. The 1 and 2 mg/kg DFP-treated group maintained these parameters at levels equal to those of the controls. The other DFP-treatment groups showed significant depressions in body weights and in food and water consumption, but these groups recovered 48 and 72 hrs after DFP administration of 3 mg/kg or 4 mg/kg, respectively. Daily injections of 2, 3 or 4 mg/kg of DFP, s.c., caused significant depressions of all three parameters, with only 75% of the 2 mg/kg group showing recovery over the 14 day treatment period. Of the 2 mg/kg group, 25% died following the 6th injection, 100% of the 3 mg/kg group were dead following the 4th injection and 100% of the 4 mg/kg group were dead following the 3rd injection. Prior to deaths in all DFP-treatment groups, the decreases in body weights and in food and water consumption were progressive and dose-dependent. The data show dose-dependency of general toxicity during acute and subacute exposure to DFP and of tolerance during subacute exposure.

Running Title: DFP Toxicity and Tolerance

Keywords: Diisopropylfluorophosphate, Toxicity, Tolerance, Rats

INTRODUCTION

The toxicity of organophosphate irreversible inhibitors of cholinesterase has been extensively studied (Murphy, 1980). Along with studies of toxicity, tolerance to these agents has aroused interest since the phenomenon was first reported by Rider et al. (1952). Those studies involved octamethyl pyrophosphoramide (OMPA) and were aimed at obtaining as much information as possible regarding the therapeutic use of OMPA in myasthenia gravis. Therefore, these studies which reported tolerance to the agent, with no gross or microscopic pathological changes, upon chronic administration, were clinically exciting. Since that report, there have been other reports of tolerance to organophosphate cholinesterase inhibitors, both in man (Summerford et al., 1953; DeRoeth et al., 1965) and in animals (Brodeur and DuBois, 1964; Glow and Richardson, 1966; Glow and Rose, 1966; McPhillips, 1969; Richardson and Glow, 1967; Stavinoha et al., 1966). Russell et al., 1971a, 1971b, 1975) and Overstreet (1973) have reported on studies of chronic administration of diisopropylfluorophosphate (DFP) and the resulting behavioral tolerance to low dose administration of DFP. In all of these reports of tolerance to DFP, an initial dose of 1.0 mg/kg of DFP to rats was followed at 3-day intervals by 0.5 mg/kg doses. Animals were housed in 24 hr lighted quarters and the one-hour consummatory behavioral responses (drinking, lapping and eating) were measured after 23 hours of food and water deprivation. Results of their studies showed not only that behavioral tolerance does develop to chronic maintenance of cholinesterase at low levels of activity (approx. 27% of normal); but they also provided evidence indicating that the time characteristics of development of tolerance to DFP was different for the different

consummatory behaviors. Development of tolerance was reported to be significantly faster as evidenced by eating behavior than by drinking behavior, the latter behavior requiring about twice as long to become normal as did eating behavior.

The present study was undertaken in order to examine more closely the effects of acute and subacute toxicities of DFP on consummatory behaviors under more natural conditions (i.e., without deprivation in addition to 12 hrs of nocturnal environment) and to further study the effects of tolerance to continuous exposure to DFP on these behaviors.

METHODS

Animals and chemicals: Male, Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) with initial weights of 175 - 200 g were used. Animals were maintained ad libitum on standard laboratory chow and tap water and were housed in a room with automatic 12 hour light and dark cycles and temperature set at 25.5 ± 1.0°C. They were kept in the animal quarters for one week prior to beginning the experiments. Diisopropylfluorophosphate (DFP), Calbiochem Lot #101318 was used throughout all studies. This preparation of DFP was found to have an IC_{50} value in vitro of 18 ,g DFP/ml (concentration required to inhibit 50% of the cholinesterase activity in whole brain homogenates from rats).

Effects of single administrations of DFP on body weight, food and water consumption: Four groups, each containing 5 rats, were injected with DFP, subcutaneously, in doses of 1, 2, 3 or 4 mg/kg in saline. A control group of 5 rats was injected with saline alone. Individual rats were kept in stainless steel metabolic cages. Twenty-five grams of powdered food and 150 ml of tap water were available for each rat every

day. Body weight and food and water consumptions of each animal were recorded daily. The percent of change from the body weight on the first day of administration was recorded for each animal. The mean body weight change (± S.E.M.) was obtained from all rats in a given group. Food and water consumptions per 100 g of body weight were calculated similarly. Differences between the means of various treatment groups were evaluated by the test of variance.

Effect of daily administration of DFP on body weight, food and water consumption, other toxicity and cumulative mortality: Three groups of eight rats each were treated daily with DFP in doses of 2, 3 or 4 mg/kg, s.c. The control group received daily s.c. injections of saline (1 cc/kg). These animals were also housed, fed, and given water exactly as described for the single injection studies. Body weights and food and water consumption of the animals in each group were recorded prior to each daily injection and other symptoms of toxicity (tremors and ocular hemorrhages) were monitored visually. The data were treated in the same way as those from the acute experiments. Any mortality appearing within a given group was recorded before administration of the next dose to survivors in that group.

RESULTS

Toxicity induced by a single administration of DFP: The results clearly demonstrate that DFP induced toxicity, as evidenced by loss of body weight, in a dose-dependent manner (Figure 1). Rats which received DFP at 3 to 4 mg/kg lost weight dramatically during the first 48 hr period following DFP administration, compared to rats treated with 1 or 2 mg/kg of DFP or with saline alone. DFP, when given at doses of 1 or 2 mg/kg, did not affect body weight. However, in the groups of rats which

received higher doses of DFP, and which did suffer weight loss, the body weights started to recover at 48 and 72 hr respectively, for the 3 mg/kg and 4 mg/kg groups. Five days after the administration of DFP, there were no significant differences in body weights between the groups.

There also were no significant changes in food consumption in the groups of animals which had been treated with single doses of 1 or 2 mg/kg of DFP (Figure 2). However, there was a dose-dependent decrease in food consumption in the groups of animals which had been administered single dose of 3 or 4 mg/kg of DFP. Rats receiving DFP in a dose of 3 or 4 mg/kg showed food consumption which was only about 40% and 20% that of the control group, respectively, at 24 hr after administration of DFP. The group which received DFP, 3 mg/kg, regained normal food consumption by 48 hours; however, the rats that received DFP, 4 mg/kg, exhibited food consumption which was still significantly reduced at this time. Interestingly, there was a significant increase in food consumption 5 days after administration of DFP, 4 mg/kg.

In terms of water consumption, rats which received the low dose of DFP (1 or 2 mg/kg) showed no difference in drinking capacity from controls; however, rats which received the higher dose of DFP (3 or 4 mg/kg) showed a 50% decrease in water consumption within 24 hr (Figure 3). Forty-eight hours after DFP administration, water consumption of these DFP-treated animals returned to normal levels. There was also evidence that rats which had been treated with 4 mg/kg DFP had significantly higher water consumption than that of the rest of the groups.

Toxicity induced by daily administration of DFP: The data in Figure 4 summarize the mortality of rats which received 3 different

doses of DFP on a daily basis. Mortality began to occur 1, 2 and 6 days after daily administration of DFP at doses of 4, 3 and 2 mg/kg, respectively. Rats which had been treated daily with high doses of DFP (3 and 4 mg/kg) all died within 4 days. Interestingly, of the rats which received DFP, 2 mg/kg, daily, seven animals survived for the two-week experimental period.

Daily administration of DFP also resulted in the loss of body weight in a dose-dependent manner (Figure 5). Rats receiving DFP at doses of 3 or 4 mg/kg lost weight dramatically compared to rats treated with 2 mg/kg of DFP or compared with saline-treated controls. The body weights of those animals which received daily administration of DFP, 2 mg/kg, steadily decreased for the first 4 days, remained steady between 4 and 8 days, and then gradually increased.

As shown in Figure 6, the high doses of DFP (3 and 4 mg/kg) caused a marked reduction in daily consumption of food. Rats receiving 2 mg/kg of DFP exhibited an initial decrease in food consumption followed by a sharp regain which continued until after ten days of daily administration of 2 mg/kg DFP; food consumption was significantly higher than that of controls.

Studies of water consumption (Figure 7) revealed the biphasic phenomenon similar to that of food consumption; that is, after the initial decrease in water consumption in the low dose DFP-treatment group, on the 9th day of daily administration of 2 mg/kg DFP, water consumption was significantly higher than that of controls.

Table 1 summarizes other toxic symptoms of rats treated daily with 2, 3 or 4 mg/kg DFP, and reveals the dose-dependency of the onset and severity of symptoms. Onset of symptoms was one day earlier in the 4

mg/kg DFP-treated group than in the 3 mg/kg group; onset of symptoms in the latter group preceeded that in the 2 mg/kg group by one day.

In both the 2 and 3 mg/kg DFP-treatment groups, tremors were noted first, preceeding ocular hemorrhages by one day. All animals in the 3 and 4 mg/kg groups died within 12 hours of the 5th and 3rd DFP doses, respectively (see Figure 4), and ocular hemorrhages were apparent in all of the animals prior to these final doses.

Ocular hemorrhages in the 2 mg/kg DFP-treated animals disappeared after the ninth dose of DFP, preceeding the disappearance of tremors by 3 days. All signs of toxicity remained absent for an additional two administrations of 2 mg/kg DFP and an additional 48 hours of observation.

DISCUSSION

The results of these studies demonstrate certain similarities to, as well as differences from other studies on DFP-induced toxicity and tolerance. It has been reported that a dosing schedule of DFP, 1 mg/kg, followed at three day intervals with doses of 0.5 mg/kg maintained brain acetylcholinesterase activity at approximately 30% of control levels (Glow and Richardson, 1966; Russell et al., 1969, 1971a,b, 1975; Overstreet, 1973). We have already reported that DFP, 1 mg/kg, daily for 14 days maintained brain acetylcholinesterase activity at approximately 33% of control activity (Sivam et al., 1982). Thus, it appears that high levels of depression of brain acetylcholinesterase activity allow for studies of DFP-induced toxicity and tolerance.

Significant decreases in both water and food intake, beginning 23 hrs after a single injection of DFP, 1 mg/kg, with short-term recovery taking place between subsequent injections of 0.5 mg/kg has been

reported (Russell et al., 1969, 1971b). It has also been reported that 24 hrs after a single injection of DFP, 1 mg/kg, brain cholinesterase activity was approximately 30% that of the normal animals (Russell et al., 1969, 1971a,b, 1975; Glow and Richardson, 1966; Glow et al., 1966; Overstreet, 1973). Although Glow et al. (1966) reported no evidence of toxic effects with brain cholinesterase activity being 20-40% of controls, they did report body weights to be significantly depressed from normal weights during chronic administration of DFP. They reported a reduction in food consumption only at days 1 and 4 (following the first and second injections of DFP, respectively) with decreased water consumption throughout the entire experimental period. Therefore tolerance to DFP in terms of water consumption was not found to occur. Russell et al. (1969, 1971b) reported tolerance to DFP-induced decreased water consumption and food consumption. Finally, Russell et al. (1969, 1971b) reported that the time to develop tolerance was shorter in terms of food intake than of drinking behavior; and furthermore that a suprabaseline drinking behavior was found following "withdrawal" of DFP and after 25 days of normal drinking.

In contrast to the above reports on DFP-induced toxicity and tolerance, we found no acute effects of a single dose of DFP, 1 mg/kg, or of twice this dose, to decrease significantly either eating or drinking behavior. After 4 daily doses of DFP, 2 mg/kg, we did observe significant depressions in both food and water consumption as well as significant depression in body weights. Concerning the level of brain cholinesterase activity 24 hr after a single dose of DFP, we have found (unpublished data) that only those doses which are lethal upon chronic administration (3 and 4 mg/kg) inhibit brain acetylcholinesterase

activity more than 50% after a single injection. We have observed significant signs and symptoms of toxicity when the enzyme activity was approximately 30% of the control level (see Table 1). Our studies agree, in part, with those of Russell et al. (1969, 1971b) in that we have found that tolerance develops to DFP in terms of both eating and drinking behaviors. However, we found that chronic DFP treatment yielded similar effects upon both of these behaviors. In fact, the data, when plotted, result in graphs which are nearly superimposable upon each other. That is, maximum decreases in both consummatory behaviors occurred at the same time. Time to develop tolerance was also the same for both behaviors as were suprabaseline performances which occurred during continuous DFP treatment.

We can only surmize that the differences between our findings and those cited above from other laboratories are due to different dosing schedules and to different potencies of DFP preparations. We have recently reported (Ho and Hoskins, 1982) on studies comparing different lots of DFP from the same and from different commercial sources. We showed that there exists some significant differences in DFP preparations such that the only way to avoid seemingly contradictory results in the literature is to report on the potency of the particular preparation of DFP used.

On the basis of those studies, we suspect that the preparations of DFP used by other laboratories were considerably more potent than the preparation used in the present study. Our own data lead us to suggest that the dose of 1 mg/kg followed at 3 day intervals by 0.5 mg/kg would be equivalent to our DFP preparations in doses of 4 mg/kg followed at three day intervals by doses of 3 mg/kg. In our hands, these doses were

lethal when given in daily injections, but not so if given intermittently.

We believe that our dosing schedule more closely relates to chronic exposure to organophosphate cholinesterase inhibitors than does intermittant dosing which, we have found (Sivam et al., 1982) does allow for some recovery of brain AChE activity (approximately 34% of inhibited AChE having regenerated by 24 hours). In the working environment, there would be continuous exposure to sublethal amounts of these agents. During chemical warfare there would most likely be acute exposure to lethal amounts of such agents. Therefore, we suggest that daily dosing with sublethal doses and acute dosing with lethal doses provides the best model for studies of toxicity from and tolerance to these agents.

Thus, we have clearly shown that tolerance does develop to sublethal continuous exposure to these agents. The tolerance can be seen in growth rates and in consummatory behavior. Furthermore, continued exposure after the tolerance has developed results in suprabaseline consummatory behaviors while growth rates remain the same as control growth rates. Many studies have provided evidence that tolerance to organophosphates is due to a depressed sensitivity of muscarinic receptors to ACh (Ehlert et al., 1980a,b; Overstreet, 1973, 1974; Russell et al., 1975; Sivam et al., 1982; Smit et al., 1980). With this in mind, then, we suggest that the suprabaseline consummatory behaviors may be due to a compensatory decrease in the activity of the sympathetic nervous system and/or of central inhibitory neurotransmitters.

Clearly, since both eating and drinking were affected similarly, our data suggest that ACh is the transmitter or neural modulator in a number of behavioral systems, including motor function and the

regulation of water intake. Thus, alteration of the cholinergic system affects all patterns of behavior which are cholinergic. Russell et al. (1969) have postulated that the motor function, eating, is less dependent upon ACh than is drinking, accounting for their finding that drinking took longer for tolerance to develop than did eating. We, on the other hand, found that with continuous exposure to DFP, tolerance developed in both behaviors at the same rate. Thus, the dependency of one behavior on ACh more than the other either does not really exist or is not related to the development of true consummatory behavioral tolerance to DFP.

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Table 1

Occurrence of Signs of Toxicity During Daily Treatment with DFP

		Number of Daily Injections											
		0	1	2	3	4	5	6	7	8	9	10	11
Group		% of Group showing toxicity											
Control	E*	0	0	0	0	0	0	0	0	0	0	0	0
	T*	0	0	0	0	0	0	0	0	0	0	0	0
DFP													
2mg/kg	E	0	0	0	0	25.0	37.5	12.5	33.3	0	0	0	0
	T	0	0	0	12.5	87.5	62.5	25.0	83.3	100	100	16.6	0
3mg/kg	E	0	0	25.0	71.4	100							
	T	0	0	50.0	100	100							
4mg/kg	E	0	0	100									
	T	0	0	100									

* E represents hemorrhages about the eye, T represents tremors.

FIGURE LEGENDS

Figure 1. Percent of original body weight (growth rates) as assessed for 8 days after a single s.c. injection of DFP. Numbers in parentheses indicate number of rats studied in each treatment group. Asterisks denote significant ($P < 0.05$) difference from saline-treated control group.

Figure 2. Food consumption by each treatment group of rats during 8 days following a single s.c. injection of DFP. Numbers in parentheses indicate number of rats studied in each treatment group. Asterisks denote significant ($P < 0.05$) difference from saline-treated control group.

Figure 3. Water consumption by each treatment group of rats during 8 days following a single s.c. injection of DFP. Numbers in parentheses indicate number of rats studied in each treatment group. Asterisks denote significant ($P < 0.05$) difference from the saline-treated control group.

Figure 4. Percent mortality in each DFP treatment group undergoing daily s.c. injections of DFP. Numbers in parentheses indicate the original number of rats in each DFP treatment group.

Figure 5. Percent of original body weight (growth rate) as assessed during daily s.c. injections of DFP over a 14 day period. Numbers in parentheses indicate original number of rats in each treatment group. Asterisks denote significant ($P < 0.05$) difference from the saline-treated control group.

Figure 6. Food consumption by each treatment group of rats during 14 days of daily s.c. injections of DFP. Numbers in parentheses indicate the original number of rats in each treatment group.

Asterisks denote significant ($P < 0.05$) difference from the saline-treated control group.

Figure 7. Water consumption by each treatment group of rats during 14 days of daily s.c. injections of DFP. Numbers in parentheses indicate the original number of rats in each treatment group. Asterisks denote significant ($P < 0.05$) difference from the saline-treated control group.

Fig 1

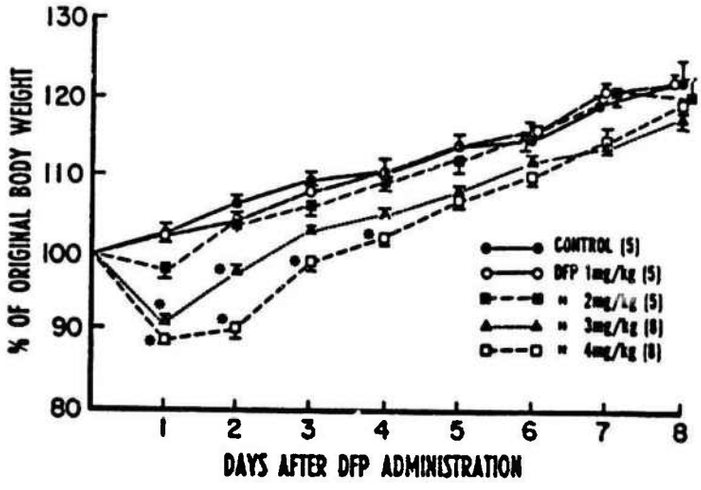

% OF ORIGINAL BODY WEIGHT
130
120
110
100
90
80
1
2
3
4
5
6
7
8
DAYS AFTER DFP ADMINISTRATION
CONTROL (5)
DFP 1mg/kg (5)
" 2mg/kg (5)
" 3mg/kg (8)
" 4mg/kg (8)

Fig. 2

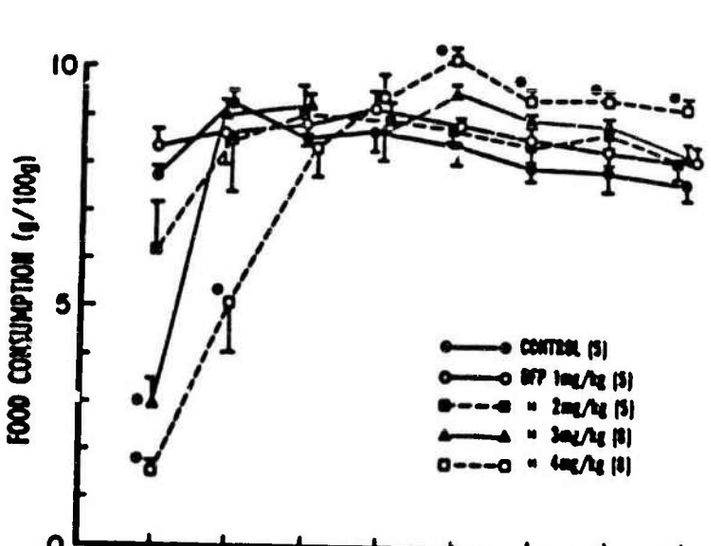

FOOD CONSUMPTION (g/100g)
10
5
0
1
2
3
4
5
6
7
8
DAYS AFTER DFP ADMINISTRATION
CONTROL (5)
DFP 1mg/kg (5)
" 2mg/kg (5)
" 3mg/kg (8)
" 4mg/kg (8)

Fig. 3

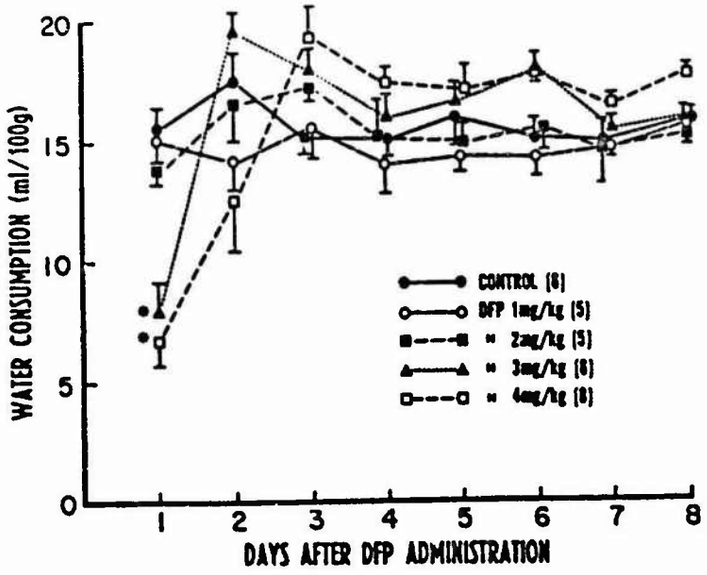

WATER CONSUMPTION (ml/100g)
20
15
10
5
0
1
2
3
4
5
6
7
8
DAYS AFTER DFP ADMINISTRATION
CONTROL (6)
DFP 1mg/kg (5)
" 2mg/kg (5)
" 3mg/kg (6)
" 4mg/kg (6)

Fig. 4

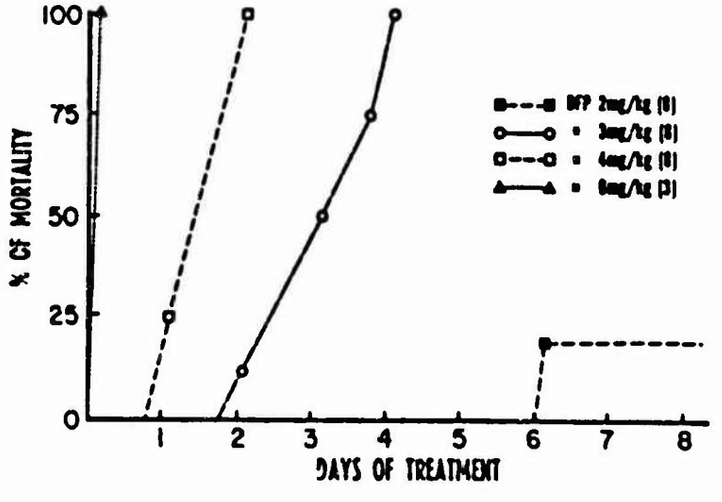

% OF MORTALITY
100
75
50
25
0
1
2
3
4
5
6
7
8
DAYS OF TREATMENT
DFP 2mg/kg (6)
" 3mg/kg (6)
" 4mg/kg (6)
" 0mg/kg (3)

Fig. 5

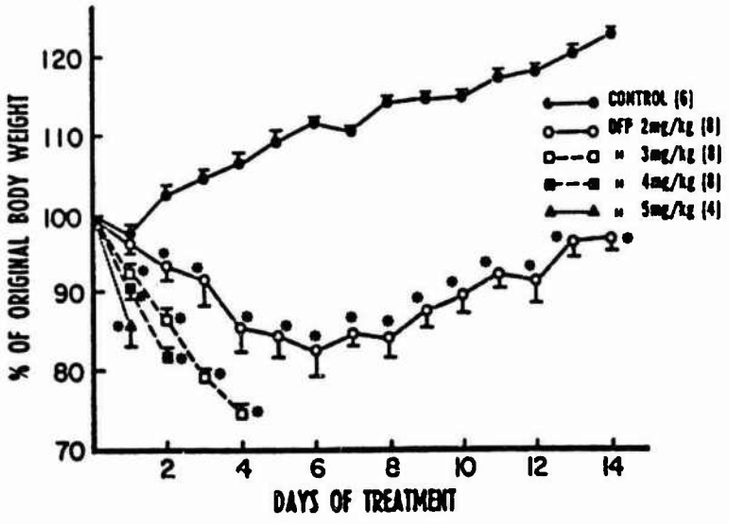

% OF ORIGINAL BODY WEIGHT
120
110
100
90
80
70
2
4
6
8
10
12
14
DAYS OF TREATMENT
CONTROL (6)
DFP 2mg/kg (8)
" 3mg/kg (8)
" 4mg/kg (8)
" 5mg/kg (4)

Fig. 6

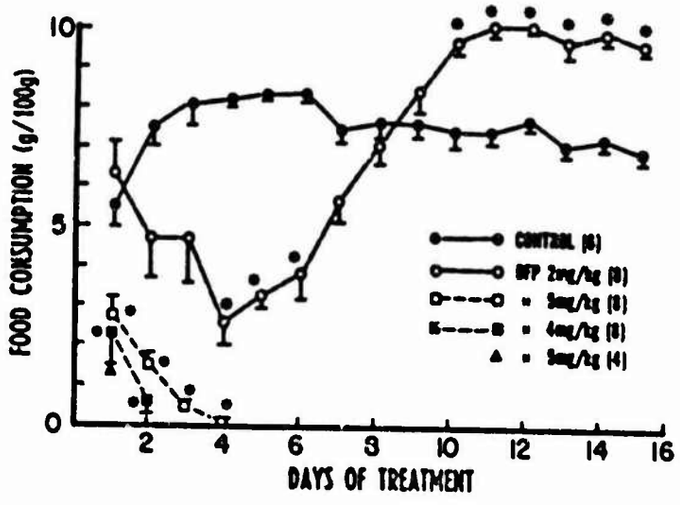
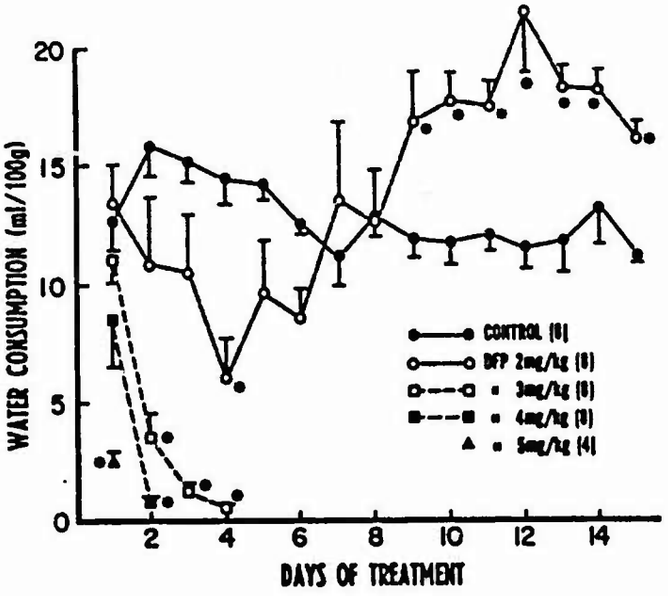

FOOD CONSUMPTION (g/100g)
10
5
0
2
4
6
8
10
12
14
16
DAYS OF TREATMENT
CONTROL (6)
DFP 2mg/kg (8)
" 3mg/kg (8)
" 4mg/kg (8)
" 5mg/kg (4)

Fig. 7


WATER CONSUMPTION (ml/100g)
20
15
10
5
0
2
4
6
8
10
12
14
DAYS OF TREATMENT
CONTROL (8)
DFP 2mg/kg (8)
3mg/kg (8)
4mg/kg (8)
5mg/kg (4)

APPENDIX 1-C

THE EFFECT OF ACUTE AND CHRONIC CHOLINESTERASE INHIBITION WITH DIISOPROPYLFLUOROPHOSPHATE ON MUSCARINIC, DOPAMINE AND GABA RECEPTORS OF THE RAT STRIATUM

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Abbreviations used: ACh, Acetylcholine; AChE, Acetylcholinesterase; B_{max}, Maximum number of binding sites; DA, Dopamine; DFP, Diisopropylfluorophosphate; GABA, Gamma-aminobutyric acid; K_D, Dissociation constant; QNB, Quinuclidinyl benzilate.

Abstract: The effects of acute and chronic administration of diisopropylfluorophosphate (DFP) to rats on acetylcholinesterase (AChE) activity (in striatum, medulla, diencephalon, cortex and medulla) and muscarinic, dopamine (DA) and GABA receptor characteristics (in striatum) were investigated. After a single injection of (acute exposure to) DFP, striatal region was found to have the highest degree of AChE inhibition. After daily DFP injections (chronic treatment), all brain regions had the same degree of AChE inhibition, which remained in a steady level despite the regression of the DFP-induced cholinergic overactivity. Acute administration of DFP increased the number of DA and GABA receptors without affecting the muscarinic receptor characteristics. Whereas chronic administration of DFP for either 4 or 14 days reduced the number of muscarinic sites without affecting their affinity, the DFP treatment caused increase in the number of DA and GABA receptors only after 14 days of treatment; however, the increase was considerably lower than that observed after the acute treatment. The in vitro addition of DFP to striatal membranes did not affect DA, GABA or muscarinic receptors. The results indicate an involvement of GABAergic and dopaminergic systems in the actions of DFP. It is suggested that the GABAergic and dopaminergic involvement may be a part of a compensatory inhibitory process to counteract the excessive cholinergic activity produced by DFP.

Key words: Acetylcholinesterase - Diisopropylfluorophosphate - Dopamine receptors - GABA receptors - Tolerance. Sivam, S. P. et al. The effect of acute and chronic cholinesterase inhibition with diisopropylfluorophosphate on muscarinic, dopamine and GABA receptors of the rat striatum.

Organophosphates constitute one of the few classes of drugs for which a mechanism of action has been defined in terms of inhibition of a specific enzyme namely acetylcholinesterase (AChE). Prior to World War II, this enzyme was of purely academic interest, but the synthesis of the organophosphorus anticholinesterase agents created new interest in the enzyme, and AChE determinations have become of practical importance to a number of people, such as those involved in the fields of industrial health, agriculture, insecticides, and chemical warfare agents (Holmstedt, 1959). In addition, accidental, suicidal and homicidal poisonings with these agents have been reported (Davis and Richardson, 1980). Organophosphates exhibit behavioral, neurological and biochemical effects in both animals and humans (Holmstedt, 1959; Davis and Richardson, 1980). On long term exposure, these compounds are known to induce neurotoxic effects (Aldridge et al., 1969; Johnson, 1975; 1977). Tolerance develops to the behavioral effects of these agents and evidence suggests that this may result from subsensitivity to acetylcholine (ACh) (Russell et al., 1969; 1981). Recent studies have demonstrated that following chronic treatment with diisopropylfluorophosphate (DFP), there is a significant decrease in the number of muscarinic cholinergic receptors labeled by ^{3}H-quinuclidinyl benzilate (^{3}H-QNB) (Schiller, 1979; Uchida et al., 1979; Ehlert et al., 1980). Enhanced cholinergic activity consequent to AChE inhibition has traditionally been believed to be the cause of many effects of organophosphates. The discovery of a variety of neurotransmitters, especially, dopamine (DA) and gamma-aminobutyric acid (GABA) and their participation in a number of neurological and behavioral disorders (Hornykiewicz, 1975; Seeman, 1981; Enna, 1981) suggest that these systems also may be influenced by organophosphates.

An imbalance of dopaminergic and cholinergic systems in the basal ganglia is associated with motor dysfunction, particularly Parkinsonism (Hornykiewicz, 1966; Hornykiewicz, 1975). The basal ganglia are rich in AChE (Quastel, 1962; Dawson and Jarrott, 1981). It is well known that GABA is an important inhibitory neurotransmitter in the mammalian CNS (Roberts et al., 1976; McBurney and Barker, 1978; Lal et al., 1980). There is evidence which suggests that the drugs which increase GABAergic activity in the brain decrease striatal DA turnover (Lahti and Losey, 1974). It has been further suggested that ACh may regulate GABA synthesis (Roberts and Hammerschlag, 1972). Finally an involvement of the GABA system in organophosphate-induced convulsions has been demonstrated (Kar and Martin, 1972; Green et al., 1977; Lundy et al., 1978). The foregoing evidence indicate that the effects produced by organophosphates include a variety of behavioral and neurological manifestations all of which cannot be attributed entirely to cholinergic hyperactivity. Other systems may, in addition or independently, be involved. The present paper describes alterations of the postsynaptic muscarinic, DA and GABA receptors, consequent to acute and chronic exposure to DFP.

MATERIALS AND METHODS

Animals

Male albino Sprague-Dawley rats (Charles River Lab, Wilmington, MA) weighing 200-250 g were used throughout the study. The animals were housed four to a cage with free access to food and water.

Administration of DFP

Freshly prepared solutions of DFP in saline (0.9%, NaCl w/v) were administered subcutaneously in volumes of 0.1 ml/100 g body weight.

Both acute and chronic treatments with DFP were carried out. In the acute treatments, a single dose of DFP 1 mg/kg or 2 mg/kg was injected and the animals were sacrificed by decapitation 6 hr, 24 hr, 7 days or 14 days after the treatment. In chronic treatments, a dose of 1 mg/kg was administered daily for 4 days or for 14 days. The treatments were scheduled in such a way that both saline treated (0.1 ml/100 g body weight) control and DFP-treated animals were sacrificed on the same day.

Determination of AChE activity

Different brain areas were dissected out according to the procedure of Glowinski and Iversion (1966). The AChE activity was determined according to the method of Ellman et al. (1961). The tissues were homogenized in ice-cold sodium phosphate buffer (0.1 M, pH 8.0) at a concentration of approximately 20 mg wet weight per ml buffer. The activities were expressed as nmoles of acetylthiocholine hydrolyzed/min/mg protein.

Membrane preparation for binding assays

Membranes were prepared according to the method of Zukin et al. (1975), with slight modification. The animals, after appropriate treatments, were decapitated, the brains were rapidly removed and the striata were dissected out. The pooled samples were then homogenized in 15 volumes of ice cold 0.32 M sucrose using a Brinkman Polytron PT-10 at low speed (setting 3). The homogenate was centrifuged at 1,000 x g for 10 min; the pellet was discarded and the supernatant fluid was centrifuged at 20,000 x g for 20 min to obtain a crude mitochondrial pellet. The crude mitochondrial pellet was resuspended in double distilled deionized water and dispersed with a Brinkman Polytron PT-10 (setting 6) for 30 sec. The suspension was centrifuged at 8,000 x g for 20 min. The supernatant including the buffy layer was collected and centrifuged at 48,000 x g for 20 min to obtain a pellet. The pellet was resuspended in

water and centrifuged at 48,000 x g for 20 min. The final pellet (membrane preparation) was suspended in Tris-HCl buffer (pH 7.4) and stored at -20°C for one to four days.

^{3}H-QNB binding

The frozen membranes were thawed and centrifuged at 25,000 x g for 15 min and the pellet was suspended in 50 mM sodium phosphate buffer (pH 7.4). The binding of ^{3}H-QNB, was carried out according to the method of Yamamura and Synder (1974), with minor modifications. In brief, the binding assay was performed in 50 mM sodium phosphate buffer (pH 7.4), with different concentrations (0.01 to 2 nM) of ^{3}H-QNB to generate saturation curves in a final volume of 1 ml. Specific binding was calculated as the total binding minus that occurring in the pressure of 1 µM atropine. The binding was initiated upon addition of 0.2 ml of membrane preparation (0.2 to 0.4 mg/ml) and incubations were allowed to proceed for 1 hr at 25°C in a shaking water bath. The reaction was terminated by rapidly filtering through Whatman GF/B glass fiber filters. Each filter was washed twice with 5 ml buffer and the dried filter was transferred to scintillation vials containing 10 ml of Aquasol (New England Nuclear, Boston, MA). The radioactivity retained in the filters was determined by liquid scintillation spectrophotometry.

^{3}H-Muscimol binding

The specific binding of the GABA receptor ligand, ^{3}H-muscimol, was carried out as previously described (Sivam et al., 1981). The frozen membrane preparation was thawed and diluted with 10 volumes of 50 mM Tris-citrate buffer (pH 7.1) and centrifuged at 25,000 x g for 20 min, to obtain a pellet; the pellet was rehomogenized with a suitable volume of the Tris-citrate buffer and incubated at 37°C for 30 min to dissociate endogenous inhibitors (Kingsbury et al., 1980). After incubation,

the suspension was recentrifuged at 25,000 x g for 20 min to obtain a pellet which was resuspended in fresh buffer for assay. Binding of ^{3}H-muscimol was initiated by the addition of 0.2 ml of the membrane preparation (1-1.5 mg/ml) to a mixture containing the required final concentration of ^{3}H-muscimol (2-100 nM) in a total volume of 1.0 ml. Incubations were carried out at 4°C for 10 min. The reaction was stopped by rapidly filtering through Whatman GF/B filters; the filters were washed twice with 5 ml of ice cold 50 mM Tirs-citrate buffer (pH 7.1) and transferred to scintillation counting vials containing 10 ml of PCS (Phase Combining System, Amersham, Co., Arlington Heights, IL). The vials were shaken for 60 min, then the radioactivity was measured by liquid scintillation spectrophotometry. Non-specific binding was determined by incubation in the presence of 1 mM unlabeled GABA.

^{3}H-Spiroperidol binding

Specific binding of the DA receptor ligang, ^{3}H-spiroperidol, was determined following the method of Tabakoff and Hoffman (1979) with minor modifications. The frozen membrane preparation was thawed and centrifuged at 25,000 x g for 15 min to obtain a pellet. The pellet was suspended in 50 mM Tris HCl (pH 7.4) containing 5 mM ethylenediaminetetracetic acid (Tris-EDTA buffer). The suspension was incubated for 20 min at 37°C and centrifuged 25,000 x g for 15 min. The supernatant was discarded and the pellet was resuspended for final assay in a suitable volume of Tris-EDTA buffer.

Aliquots of (0.2 ml) of the membrane preparation (2-2.5 mg/ml) were incubated with various concentrations (0.05 to 2 nM) of ^{3}H-spiroperidol in the presence of 10^{-7} M (+)- or (-)-butaclamol (Seeman et al., 1976),

in a final volume of 1 ml. Incubations were carried out for 20 min at 25°C. At the end of the incubation, the sample were filtered under reduced pressure through glass fiber filters (GF/B, Whatman). The filters were then washed with 5 ml of Tris-EDTA buffer and after drying, were placed into scintillation vials with 10 ml of Aquasol. After at least 45 min of shaking to equilibrate, the radioactivity was quantified by liquid scintillation spectrophotometry. Specific binding of spiroperidol was defined as the amount bound in the presence of 10^{-7} M (-)-butaclamol minus that bound in the presence of 10^{-7} M (+)-butaclamol.

In vitro effect of DFP on receptor binding

The effect of in vitro additions of DFP, (10^{-9} to 10^{-4} M) upon binding of ^{3}H-QNB (0.2 nM), ^{3}H-muscimol (5 nM) or ^{3}H-spiroperidol (0.2 nM) was investigated using the methods described above.

Determination of protein concentration

The protein content of the membrane preparations was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Drugs and chemicals

^{3}H-QNB (specific activity, 40.2 Ci/mmol), ^{3}H-muscimol (specific activity, 10.1 Ci/mmol) and ^{3}H-spiroperidol (specific activity, 31.3 Ci/mmol) were obtained from New England Nuclear Corporation, Boston, MA. The radiochemical purity was verified by thin layer chromatography using appropriate solvent systems. DFP (Lot #103288) was obtained from Calbiochem, LaJolla, CA; this preparation of DFP was found to have an IC_{50} (concentration required for 50% inhibition of control AChE activity (in the whole brain homogenate of rat) value in vitro of 12 µg/ml. Atropine

sulphate and GABA were obtained from Sigma Chemical Company, St. Louis, MO. Dextro (+) and levo (-)-butaclamol were generously supplied by Ayerst Laboratories, New York, NY. Other chemicals and reagents of analytical grade were obtained from commercial suppliers.

Statistics

Linear regression analyses were used to obtain all values of dissociation constant (K_D) and maximum number of binding sites (B_{max}). When applicable, data were analyzed for significance by Student's t-test: a P value of < 0.05 between two means was considered significant.

RESULTS

Effect of DFP on the gross behavioral activity, and on AChE activity in different regions of the rat brain

Acute administration of DFP in 1 or 2 mg/kg doses, induced parasympathetic overactivity consisting of salivation, lacrimation, urination, excessive bronchial secretion, sweating, diarrhea, muscular twitching, fasciculations and tremor. On chronic administration of DFP (1 mg/kg, daily) the animals continued to exhibit these symptoms for 4 to 5 days; thereafter, the symptoms regressed and by the 14th day, the animals no longer showed any of the symptoms including the characteristic intermittant tremor observed on the 4th and 5th day. The relative activity of AChE in different regions of brains from control rats was as follows: striatum > medulla $\simeq$ diencephalon > cortex $\simeq$ cerebellum (Table 1). Six hr after an acute administration of DFP (1 mg/kg), AChE activity was significantly reduced in all brain regions except the cerebellum. Striatum and medulla were affected more than the other regions. When the dose of DFP was increased to 2 mg/kg, the enzyme activity was about 20% of control in striatum, diencephalon and medulla,

whereas, in the cortex and cerebellum, the activity was 38 and 75%, respectively.

Twenty-four hr after administration of 1 mg/kg DFP, the inhibition of AChE activity remained almost the same as that observed 6 hr after DFP administration. The relative activities among different regions were similar to those in control brains, despite inhibition. Twenty-four hr after administration of 2 mg/kg DFP, the enzyme activity had recovered significantly ($P < 0.05$) in straitum, medulla and diencephalon compared to the activities in these areas, 6 hr after administration of the same dose. In cortex and cerebellum, the inhibition 24 hr after DFP administration was not different from that observed 6 hr after administration. The inhibition 24 hr after a single injection of 2 mg/kg was in general, the same as that observed 6 hr after a single injection of 1 mg/kg DFP.

After chronic administration of DFP for 4 and 14 days, there was a substantial inhibition of AChE in all regions (Table 2). The degree of inhibition after 4 days of daily treatment with 1 mg/kg DFP was significantly higher than that observed after a single DFP injection. The levels of AChE activity after 4 and 14 days of daily DFP treatment were essentially the same.

Effect of DFP on muscarinic receptor binding

Scatchard analysis of ^{3}H-QNB binding data revealed a single population of muscarinic receptors. A single injection of DFP (1 or 2 mg/kg) failed to alter either the K_D or the B_{max} except for a 20% decrease in the latter 24 hr after DFP treatment (Table 3). After chronic treatment with DFP for 4 and 14 days, the B_{max} was significantly decreased (Table 4; Fig. 1).

None of the treatments affected the affinity of muscarinic receptors.

Effect of DFP on GABA receptor binding

Six hr after an acute treatment with DFP (1 mg/kg), the number of striatal GABA receptors, as determined by Scatchard analysis, were increased by 37%; 24 hr after the treatment, the receptor population had returned to control levels (Table 3; Fig. 2). On the other hand, DFP at a dose of 2 mg/kg increased the number of GABA receptors both 6 and 24 hr following treatment, though there was a significant ($P < 0.05$) difference between these groups. Seven days after a single injection of DFP (1 mg/kg), there was a significant decrease in GABA receptor population. Daily administration of DFP (1 mg/kg) for 4 and 14 days showed a significant increase only in the latter group (Table 4). Neither acute nor chronic administration of DFP altered the affinity of GABA receptors.

Effect of DFP on DA receptor binding

Saturation studies with ^{3}H-spiroperidol as a ligand and Scatchard analysis of the data revealed a single population of DA receptors. Six and 24 hr after a single injection of DFP (1 or 2 mg/kg), an increase in the B_{max} of ^{3}H-spiroperidol binding (Table 3; Fig. 3) was observed. There was a significant ($P < 0.05$) difference between the increase produced by 1 and 2 mg/kg DFP after 6 hr; however 24 hr after these doses, the increases in the DA populations induced by the two doses did not differ significantly. Daily treatment with 1 mg/kg DFP for 4 days had no effect on the number of DA receptors, whereas DFP treatment for 14 days produced a significant increase in the DA receptor population. This increase was significantly ($P < 0.05$) lower than that observed 24 hr after a single injection of DFP at a dose of 1 or 2 mg/kg (Table 4).

None of the acute or chronic DFP treatments affected the K_D of ^3H-spiroperidol.

Effect of in vitro addition of DFP on receptor binding

The in vitro presence of DFP at concentrations ranging from 10^{-9} to 10^{-4} M, failed to affect muscarinic, GABA or DA receptor binding.

DISCUSSION

Although there have been many studies on brain AChE inhibition by DFP and related agents, relatively few have reported studies of discrete brain areas. In brains from control animals, the relative activity of AChE in different regions indicated that straitum had the highest activity followed by medulla, diencephalon, cortex and cerebellum. This is consistent with the reports of other workers (Silver, 1974; Dawson and Jarrott, 1980). All regions were not, in general, uniformly affected by a single injection of the low dose (1 mg/kg) of DFP, in contrast to the effects of single high dose (2 mg/kg) treatment which affected all regions equally. For example, striatum, medulla and diencephalon were affected to a larger extent and the degree of inhibition was similar among these regions. The cortex was affected to a lesser extent and the cerebellum was not affected by low dose acute treatments. It is possible that the low levels of basal AChE activities of the cortex and cerebellum may be related to this observation. It should be pointed out that the inhibition of AChE after a single low dose was about 50% at both 6 and 24 hr treatment. After continuous administration of DFP, a steady level of inhibition (65%) was maintained during the treatment period. Further, during chronic exposure, all regions of the brain were equally susceptible to AChE inhibition, unlike

acute exposure. Moreover, continuous administration did not lead to a recovery of enzyme activity. This confirms earlier reports that AChE remained chronically depressed despite the fact that behavioral tolerance readily occurs during chronic treatment (Russell et al., 1969; Chippendale et al., 1972; Kozar et al., 1976; Russell et al., 1981). Since behavioral tolerance developed during this period of enzyme inhibition, the behavioral effects cannot be attributed entirely to AChE inhibition.

It has been suggested that the behavioral tolerance which occurs during chronic administration of DFP, may result from a decreased number of muscarinic receptors (Bignami et al., 1975; Russell et al., 1975). In the present study, a reduction of muscarinic receptor density was observed during chronic treatment with DFP. Thus our data confirm other reports (Bull, 1965; Costa et al., 1981; Ehlert et al., 1980; Gazit et al., 1979; Gokhale et al., 1977; Levy, 1981; Schiller, 1979; Uchida et al., 1979) and provide direct confirmation of the viewpoint that a reduction in the number of muscarinic receptors may be responsible, at least in part, for the development of tolerance to DFP. It is of interest that Dawson and Jarrott (1981), while reporting little change in muscarinic receptors in guinea pigs, during acute or chronic administration of DFP did observe behavioral tolerance to chronic DFP treatment. It remains to be confirmed whether this is due to species difference.

The present study indicates that the reduction in muscarinic receptor density is time-dependent; 4 days of treatment caused a 43% reduction, whereas 14 days of treatment resulted in a 61% reduction from control values.

This was despite the fact that AChE activity remained inhibited to the same extent after 4 or 14 days of DFP treatment. Therefore, the reduction in the number of muscarinic receptors appears to be a gradual process not directly related to the degree of AChE inhibition. This is consistent with our observation and that of Russell et al. (1975) that the DFP-induced cholinergic effects such as diarrhea, tremor, salivation, and lacrimation disappear gradually.

It is not clear whether all of the toxic effects of DFP are due to alterations in cholinergic function or if other neurochemical changes might be involved. In the present study, the numbers of both GABA and DA receptors were significantly increased after acute treatment, but the increases were less prominent after chronic treatment. It has been reported that anticonvulsants, which are believed to act primarily via GABAergic mechanisms, block organophosphate induced convulsions (Lipp, 1973; Rump et al., 1973; Lundy and Magor, 1977). Lundy and Magor (1977) and Lundy et al. (1978) showed that small amounts of benzodiazepines, which are believed to act through enhancing GABAergic transmission (Olsen, 1981)] totally abolished organophosphate-induced convulsions, whereas, the antimuscarinic agent, atropine, had no effect, even in high doses. These authors further showed that amino-oxyacetic acid (AOAA), which elevates GABA levels by inhibiting GABA-transaminase, also inhibited organophosphate-induced convulsions. Kar and Martin (1972) suggested that paraoxon convulsions are related to GABA levels in the central nervous system. Certain organophosphates cause convulsions and death but do not inhibit AChE (Bellet and Casida, 1976) and are believed to produce their effects by altering central GABA function (Bowery et al., 1976). Involvement of GABA in a variety of neuropsychiatric

disorders including epilepsy and schizophrenia has been documented (Enna, 1981). The evidence, therefore, seems to indicate that GABA receptor activation may play a part in the acute effects of organophosphates.

In our study, we observed that, not only GABA receptor density, but also DA receptors density was increased after a single injection of DFP. Others have observed that DA levels are increased after acute DFP treatment (Gilisson et al., 1974). On the other hand, mipafox was found to decrease DA levels after chronic administration (Freed et al., 1976). The increased motor activity of Parkinsonism is known to be due to an imbalance of cholinergic and dopaminergic activity in the basal ganglia, i.e., increased cholinergic activity due to DA deficiency (Weiss et al., 1976; Heibronn and Bartfai, 1978). It has been reported that striatal DA has an inhibitory effect on striatal neurons (Krnjevic and Phillis, 1963) and also reduces the spontaneous and cholinergic neuronal firing in the striatum (McGreer et al., 1975). It has been suggested that dopaminergic (inhibitory) and cholinergic (excitatory) mechanisms interact in a delicate way to maintain the normal function of striatum (Anden et al., 1966). The striatal increases in GABA and DA receptor densities observed after acute treatments with DFP, returned gradually to control levels after cessation of treatment. Thus the neurochemical imbalance produced as a result of acute inhibition of AChE may be partially counteracted by an acute increase in dopaminergic activity supported by an increase in GABAergic activity.

The changes observed in the receptor bindings were not due to a direct effect of DFP on muscarinic, DA or GABA receptors, since no inhibition of binding was observed by high concentration (10^{-4} M) of DFP when added in vitro.

This confirms the earlier report of Ehlert *et al.* (1980) who showed that the muscarinic receptors were not affected by high concentrations (10^{-5} M) DFP added *in vitro*.

The results of our study indicate an involvement of ACh, DA and GABA receptors in the effects of organophosphates. It is suggested that the GABA and DA systems, singularly or in combination, counteract the enhanced cholinergic activity induced by organophosphates.

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TABLE 1. Effect of acute administration of DFP on AChE activity in the discrete regions of rat brain

Treatment	AChE activity (nmoles/min/mg protein)				
	Striatum	Medulla	Diencephalon	Cortex	Cerebellum
Control[a]	186 ± 9	87 ± 7	75 ± 6	29 ± 4	32 ± 2
1 mg/kg, DFP (single dose)					
After 6 hr[a]	88 ± 5*** (53)	36 ± 2** (59)	45 ± 6* (40)	17 ± 2* (40)	28 ± 4 (15)
24 hr[a]	95 ± 6** (49)	43 ± 4** (51)	42 ± 4* (43)	18 ± 3* (37)	27 ± 2 (16)
7 days[b]	130 ± 5* (30)	57 ± 6* (37)	55 ± 4* (27)	25 ± 3 (15)	29 ± 3 (11)
14 days[b]	162 ± 13 (13)	78 ± 4 (10)	64 ± 7 (15)	28 ± 4 (3)	30 ± 4 (6)
2 mg/kg, DFP (single dose)					
After 6 hr[a]	29 ± 2*** (84)	25 ± 2*** (74)	14 ± 2*** (81)	11 ± 2** (62)	24 ± 2* (25)
24 hr[a]	84 ± 3*** (55)	37 ± 3** (57)	28 ± 3** (62)	12 ± 2* (59)	23 ± 2* (29)
7 days[b]	117 ± 8* (37)	62 ± 8* (29)	45 ± 5* (40)	22 ± 3* (25)	27 ± 3 (15)
14 days[b]	130 ± 8* (30)	65 ± 6* (26)	56 ± 3* (27)	25 ± 2 (14)	29 ± 4 (10)

Values represent mean ± S.E. of at least three samples. Numerals in the parenthesis denote % inhibition from the control values. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the respective control values.
[a]n = 4
[b]n = 3

TABLE 2. Effect of chronic administration of DFP on AChE activity in different regions of rat brain

Treatment	AChE activity (nmoles/min/mg protein)									
	Striatum		Medulla		Diencephalon		Cortex		Cerebellum	
Control[a]	195 ± 6		85 ± 4		73 ± 5		33 ± 3		34 ± 3	
1 mg/kg, daily 4 days[b]	61 ± 3***	(72)	31 ± 2**	(65)	38 ± 3**	(48)	15 ± 2**	(55)	12 ± 1**	(63)
Control[a]	187 ± 9		92 ± 4		74 ± 3		29 ± 3		35 ± 2	
1 mg/kg, daily 14 days[b]	44 ± 6***	(76)	25 ± 3***	(67)	25 ± 2***	(66)	13 ± 1**	(57)	11 ± 2***	(68)

Values represent mean ± S.E. of at least 4 samples. Numerals in parenthesis denote % inhibition from the respective control values. **$p < 0.01$, ***$p < 0.001$ compared to the respective control values.
[a] $n = 4$,
[b] $n = 3$.

TABLE 3. Effect of acute administration of DFP on ^{3}H-QNB, ^{3}H-muscimol and ^{3}H-spiroperidol binding in the rat striatum

Treatment	^{3}H-QNB		^{3}H-muscimol		^{3}H-spiroperidol	
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}
Control	0.076 ± 0.012	1237 ± 53 (4)	7.2 ± 0.9	382 ± 17 (4)	0.114 ± 0.012	223 ± 16 (4)
1 mg/kg DFP						
After 6 hr	0.122 ± 0.062	1097 ± 59 (3)	8.3 ± 0.4	525 ± 19**(4)	0.135 ± 0.031	350 ± 12**(4)
24 hr	0.087 ± 0.006	1314 ± 82 (3)	6.7 ± 0.5	393 ± 22 (3)	0.119 ± 0.042	401 ± 13**(3)
7 days	0.093 ± 0.007	1135 ± 79 (3)	9.2 ± 0.9	279 ± 21* (3)	0.143 ± 0.052	243 ± 21 (3)
14 days	0.075 ± 0.009	1153 ± 68 (3)	8.5 ± 0.7	337 ± 17 (3)	0.123 ± 0.051	193 ± 18 (3)
2 mg/kg DFP						
After 6 hr	0.063 ± 0.032	1345 ± 83 (3)	10.2 ± 0.9	566 ± 18**(4)	0.115 ± 0.013	433 ± 24**(4)
24 hr	0.094 ± 0.019	989 ± 29*(3)	7.5 ± 0.5	501 ± 19**(4)	0.119 ± 0.015	429 ± 20**(4)
7 days	0.076 ± 0.008	1023 ± 43 (3)	8.8 ± 0.6	392 ± 10 (3)	0.144 ± 0.054	210 ± 13 (3)
14 days	0.088 ± 0.023	1102 ± 38 (3)	7.8 ± 0.7	367 ± 11 (3)	0.134 ± 0.063	256 ± 23 (3)

K_D = nM; B_{max} = pmole/g protein. The K_D's and B_{max}'s were derived from Scatchard analysis of the binding data. The binding was carried out by a filtration method as described in "Materials and Methods". The values are the mean ± S.E. of three to four determinations each done in duplicate (^{3}H-QNB binding) or triplicate (^{3}H-muscimol and ^{3}H-spiroperidol). Numerals in parenthesis denote the number of determinations. *$P < 0.05$; **$P < 0.01$ compared to control.

TABLE 4. Effect of chronic administration of DFP on ^{3}H-QNB, ^{3}H-muscimol and ^{3}H-spiroperidol binding in the rat striatum

Treatment	^{3}H-QNB		^{3}H-muscimol		^{3}H-spiroperidol	
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}
Control	0.065 ± 0.006	1207 ± 57 (3)	6.1 ± 0.4	452 ± 27 (3)	0.168 ± 0.085	198 ± 15 (3)
1 mg/kg, daily x 4 days	0.056 ± 0.004	684 ± 25***(3)	8.2 ± 0.3	513 ± 15 (3)	0.143 ± 0.023	220 ± 14 (3)
Control	0.064 ± 0.005	1323 ± 53 (3)	8.1 ± 0.8	393 ± 21 (3)	0.121 ± 0.042	232 ± 18 (3)
1 mg/kg, daily x 14 days	0.066 ± 0.006	510 ± 36***(4)	7.3 ± 0.7	492 ± 41*(4)	0.124 ± 0.033	295 ± 16*(4)

K_D = nM; B_{max} = pmole/g protein. The K_D's and B_{max}'s were derived from Scatchard analysis of the binding data. The binding was done by a filtration method as described in "Materials and Methods". The values are the mean ± S.E. of three to four determinations each in duplicate (^{3}H-QNB binding) or triplicate (^{3}H-muscimol and ^{3}H-spiroperidol binding). Numerals in parenthesis denote the number of determinations. *P < 0.05; ***P < 0.001 compared to the respective control.

LEGENDS FOR FIGURES

Fig. 1. Effect of chronic administration of DFP on ^{3}H-QNB binding in the striatal homogenates of rat. Incubations were carried out for 60 min at 24°C, followed by filtration. Non-specific binding was performed in the presence of 1 μM atropine.

Fig. 2. Effect of acute administration of DFP on ^{3}H-muscimol binding in the striatal homogenates of rat. Incubations were carried out for 10 min at 4°C, followed by filtration. Non-specific binding was performed in the presence of 1 mM GABA.

Fig. 3. Effect of acute administration of DFP on ^{3}H-spiroperidol binding in the striatal homogenates of rat. Incubations were carried out for 20 min at 25°C, followed by filtration. The specific binding denotes the difference between the binding in the presence of 10^{-7} M (+)- and (-)-butaclamol.

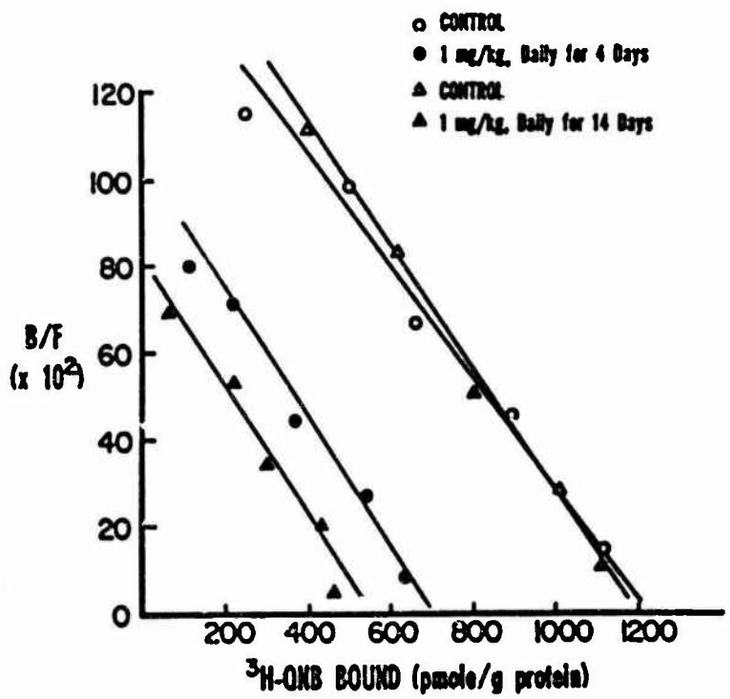
CONTROL
1 mg/kg, Daily for 4 Days
CONTROL
1 mg/kg, Daily for 14 Days
B/F
(x 10²)
120
100
80
60
40
20
0
200
400
600
800
1000
1200
³H-QNB BOUND (pmole/g protein)

Fig. 1

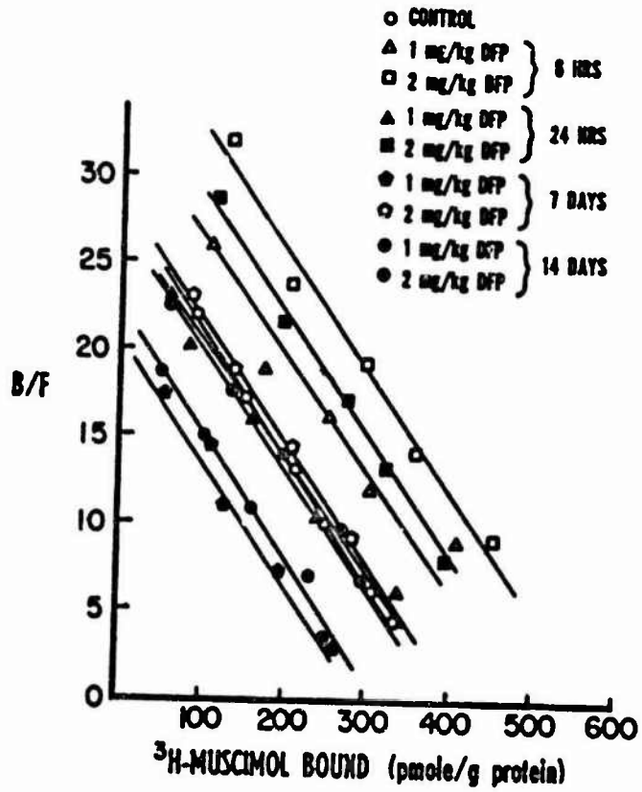
CONTROL
1 mg/kg DFP
2 mg/kg DFP
8 HRS
1 mg/kg DFP
2 mg/kg DFP
24 HRS
1 mg/kg DFP
2 mg/kg DFP
7 DAYS
1 mg/kg DFP
2 mg/kg DFP
14 DAYS
B/F
0
5
10
15
20
25
30
100
200
300
400
500
600
3H-MUSCIMOL BOUND (pmole/g protein)

Fig. 2

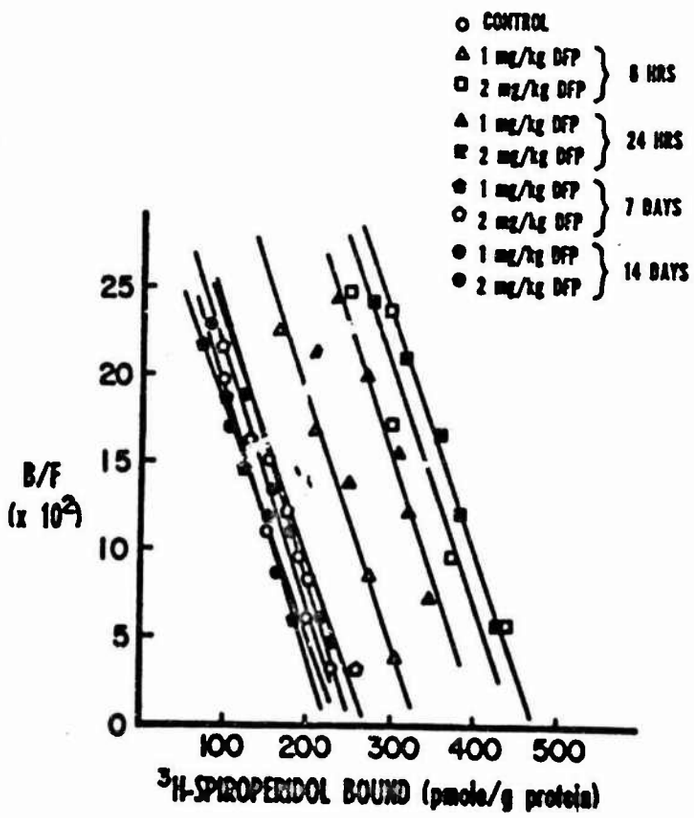
CONTROL
1 mg/kg DFP
2 mg/kg DFP
8 HRS
1 mg/kg DFP
2 mg/kg DFP
24 HRS
1 mg/kg DFP
2 mg/kg DFP
7 DAYS
1 mg/kg DFP
2 mg/kg DFP
14 DAYS
B/F
(x 10³)
25
20
15
10
5
0
100
200
300
400
500
³H-SPIROPERIDOL BOUND (pmole/g protein)

Fig. 3

APPENDIX 1-D

DIISOPROPYLFLUOROPHOSPHATE AND GABA SYNAPTIC FUNCTION: EFFECT ON LEVELS, ENZYMES, RELEASE AND UPTAKE IN THE RAT STRIATUM

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Summary:

The effects of acute (single dose, 1 or 2 mg/kg, s.c.) and sub-acute (1 mg/kg, s.c., daily for 4 or 14 days) administration of diisopropylfluorophosphate (DFP) to rats on cholinergic enzymes, acetylcholinesterase (AChE), choline acetyltransferase (CAT) and on GABA synaptic function were investigated in the striatal region of brain. Acute and sub-acute administration of DFP inhibited AChE activity but did not affect CAT activity. The cholinergic hyperactivity seen on acute administration receded gradually during continuous treatment, despite a steady inhibition of AChE activity during this period. In general, acute as well as sub-acute treatments increased levels of GABA and its precursor (glutamate) and decreased GABA uptake and release. However, none of the treatments affected activities of the GABA related enzymes: glutamic acid decarboxylase and GABA-transaminase. These results, together with our previous finding that the postsynaptic GABA receptor population is increased by DFP treatment, indicate that GABA transmission is affected by DFP. An overall enhancement of GABA function appears to be involved in DFP poisoning; it is suggested that this may be a compensatory mechanism to counteract the cholinergic hyperactivity.

Key words: Diisopropylfluorophosphate, tolerance, acetylcholinesterase, GABA system

Introduction:

Organophosphates are known to exhibit a broad spectrum of behavioral, neurological and biochemical effects in both animals and humans[1,4,12,13]. An enhanced cholinergic overactivity consequent to the inhibition of the enzyme acetylcholinesterase (AChE) which terminates the activity of the cholinergic transmitter acetylcholine (ACh), has been traditionally and generally believed to be the cause of the many effects of organophosphates. The relatively, recent awareness of the function of a variety of central nervous system (CNS) neurotransmitters, especially, γ-aminobutyric acid (GABA) and its alleged participation in a variety of neurological and behavioral disorders, raises the possibility of an involvement of GABA in organophosphate toxicity. It is well recognized that GABA is an important inhibitory neurotransmitter in the mammalian CNS [15,22,27]. There is evidence which suggests that acetylcholine may regulate GABA synthesis[26]. An involvement of GABA has been suggested in Paraoxon[14]- and soman[17,20]-induced convulsions. The foregoing evidence indicate a possible interaction between the cholinergic and GABA systems. The present study deals with the acute and sub-acute effect of diisopropylfluorphosphate (DFP) on various steps of GABA synaptic function.

Methods:

Animals and Chemicals:

Male Sprague-Dawley rats (Charles River Lab, Wilmington, MA) weighing 175-250 g were used in the present study. Animals were

maintained ad libitum on standard laboratory chow and tap water and were housed in a room with automatic 12 hour light and dark cycles and temperature set at 25.5 ± 1.8°C. Diisopropylfluorophosphate (DFP, Lot #103288; Calbiochem, La Jolla, CA) was used throughout all studies. This preparation of DFP was found to have an IC_{50} value in vitro of 12 µg DFP/ml (concentration required to inhibit 50% of the cholinesterase activity[6] in homogenates of whole brains from rats). L-[^{14}C(U)]-glutamic acid (271 mCi/mmol), ^{3}H-GABA (33·3 Ci/mmol) and [^{14}C]-acetyl CoA (53 mCi/mmol) were obtained from New England Nuclear, Boston, MA. The radiochemical purity was verified by thin layer chromatography using appropriate solvent systems. Acetylthiocholine iodine and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Calbiochem-Behring, La Jolla, CA. Acetyl-CoA, GABA, tetraphenylboron, glutamic acid, 3,5-diaminobenzoic acid, succinic semialdehyde, pyridoxol phosphate, glutathione, α-ketoglutaric acid, NADH, NADP, NADPH, GABAase, 2-mercaptoethanol, glycine and hydrazine hydrate were obtained from Sigma Chemical Co., St. Louis, MO. Glutamate dehydrogenase was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. Other chemicals and reagents were obtained from commercial suppliers.

Administration of DFP:

Stock solutions (10 mg/ml) of DFP were prepared in normal saline (0.9%, NaCl) and kept frozen at -30°C. Just before injection, the stock solution was diluted in saline to give the desired concentrations. DFP was administered subcutaneously in a

volume of 0.1 ml/100 g body weight. Both acute and sub-acute treatment with DFP were carried out between 9-11 a.m. everyday. In the acute treatments, a single dose of DFP, 1 or 2 mg/kg was injected and the animals were sacrificed by decapitation 6 hr, 24 hr, 7 days or 14 days after the treatment. The entire treatment was scheduled in such a way that both control and treated animals were sacrificed on the same day. In the sub-acute treatments, a single dose of DFP, 1 mg/kg, was administered daily for 4 or 14 days. Appropriate controls received saline (0.1 ml/100 g body weight).

Dissection of striatum:

The striata of the brain were dissected out following the method of Glowinski and Iversen[10]. A microwave oven (Thermex #44104 Metabostat System, Santa Clara, CA) was used to irradiate the head for 2 sec at 2.5 kw, for the determination of GABA and glutamate levels .

Determination of AChE activity:

The determination of AChE activity was carried out according to Ellman et al[6]. In brief, the homogenates were prepared in ice-cold 0.1 M sodium phosphate buffer, pH 8.0. The enzyme activity was measured photometrically by measuring the intensity of the yellow color produced from acetylthiocholine when it reacts with dithiobisnitrobenzoate ion. The AChE activity was expressed as nmoles acetylthiocholine hydrolyzed/min/mg protein.

Determination of choline acetyltransferase (CAT) activity:

The radiochemical assay developed by Fonnum[8] was used to assay CAT activity. In brief, homogenates of striatum were prepared in 10 mM EDTA buffer, pH 7.4. The incubation mixture contained (final concentration): 0.2 mM acetyl-CoA containing 0.5 μM [1-^{14}C]acetyl-CoA, 300 mM NaCl, 50 mM sodium phosphate buffer (pH 7.4), 8 mM choline chloride, 20 mM EDTA buffer (pH 7.4) and 0.1 mM physostigmine. The homogenate (0.2 ml) was incubated with 0.8 ml of the incubation mixture for 15 min at 37°C. At the end of the incubation the contents of the reaction vessels were transferred to scintilation vials. The tubes were washed once with 5 ml of sodium phosphate buffer (10 mM, pH 7.4) and the wash was added to the scintillation vials. To each vial, ml of acetonitrile containing 10 mg of tetraphenylboron and 10 ml of toluene scintillation mixture [0.05% diphenyloxazole and 0.02% 1,4-bis-(4-methyl-5-phenyloxazole-2-yl) benzene] were added. The vials were shaken gently for 1 min and then the organic and aqueous layers were allowed to separate for 10 min. radioactivity present in the organic layer was counted by liquid scintillation spectrometry. Enzyme activity was expressed as nmole ACh formed/hr/mg protein.

Determination of GABA-transaminase (GABA-T) activity:

GABA-T activity was measured following the procedure of Löscher[18], based on the method of Salvador and Albers[34]. In brief, the assay consisted of fluorometic measurement of succinic

semialdehyde produced from GABA during incubation with GABA-T. The incubation medium contained in a final volume of 1.0 ml: 50 mM α-ketoglutaric acid, 125 mM GABA and 0.2 ml of homogenate. The samples were incubated at 37°C for 60 min, and then cooled in an ice bath and centrifuged at 3500 x g for 10 min at 4°C. An aliquot (0.3 ml) of the supernatant was added to 0.3 ml of 200 mM 3,5-diaminobenzoic acid and the samples were heated at 60°C for 1 hr. After dilution 1: 100 or 200, the samples were activated at 405 nm in a spectrophotofluorometer and the fluorescence was read at 505 nm. The enzyme activity was expressed as nmoles succinic semialdehyde formed/hr/mg protein.

Determination of glutamic acid decarboxylase (GAD) activity:

GAD activity was assayed as previously described[28]. Homogenates were prepared in 0.1 M potassium phosphate buffer (pH 6.5). The incubation mixture (final volume 1 ml) contained 10 μM glutamate which include 100 nM of (U-^{14}C)-L-glutamate, 0.2 mM pyridoxol phosphate and 1 mM glutathione. Two tenths ml of the homogenate and 0.8 ml of the incubation mixture were placed in a test tube and closed with a rubber stopper, from which hung a plastic vial containing 1 M hyamine hydroxide in methanol. The reaction was carried out at 37°C for 30 min in a water bath. At the end of the incubation 0.2 ml of 4N HSO_4 was injected through the rubber stopper along the inner side of the test tube in order to stop the reaction and release CO. After equilibrating at 37°C for 90 min, the plastic vials were removed and placed in a scintillation counting vial with 10 ml of Aquasol (New England

Nuclear). The radioactivity was measured by a liquid scintillation spectrometry. The enzyme activity was expressed in nmoles ^{14}C-CO released/hr/mg protein.

Determination of GABA and glutamate levels:

The GABA and glutamate levels were measured by the fluorometric method described by Graham and Aprison[11]. Briefly, the brain tissue was homogenized in 75% ethanol. After centrifugation, the supernatant was air-dried, the residue was suspended in water; the clear supernatant obtained after ultracentrifugation of the suspension was used for GABA and glutamate determination. For GABA determination, the reaction mixture (final volume 0.4 ml) contained 3 mM α-ketoglutarate, 1.5 mM 2-mercaptoethanol, 0.4 mM NADP, and 1 unit/ml of GABAase. Incubations were carried out at 37°C for 50 min and the reaction was stopped by heating the samples at 60°C for 30 min. The NADPH formed was measured fluorometrically (excitation 350 nm and emission 460 nm). The glutamate concentration was measured as follows: the reaction mixture contained in glycine-hydrazine buffer (5 mM, pH 8.6), 0.2 mM NAD and 100 µg of glutamate dehydrogenase from beef liver. The fluorescence was measured in a spectrophotofluorometer at 350 nm excitation and 460 nm emission.

^{3}H-GABA uptake in crude synaptosomal preparations of striatum:

Animals were sacrificed by decapitation and the brains were removed and the striata were dissected out. Striata from rat brains were pooled and homogenized in 20 volumes of 0.32 M sucrose, using a Poller-Elvejem glass homogenizer. The homogenate was centrifuged at 1,000 g for 10 min at 4°C. The supernatant thus obtained, was centrifuged at 20,000 g for 20 min at 4°C to obtain a pellet (P fraction). The pellet, after resuspension in 0.32 M sucrose, was used in the uptake studies.

^{3}H-GABA uptake was measured using the method of Olsen et al[24]. The pellet suspension (0.2 ml) was incubated for 30 min at 0°C in a medium containing 0.1 M NaCl, 0.1 mM aminooxyacetic acid (AOAA), and 0.05 M Tris HCl buffer, pH 7.4. The ^{3}H-GABA concentration varied from 12.5 to 150 nM. Non-specific uptake was determined in the presence of 1 mM unlabelled GABA. Triplicate samples (final volume of 1 ml) were used. The protein concentration was 0.5-1.0 mg/ml. After the incubation, the reaction mixture was filtered under vacuum with GF/B glass filters, washed twice with 5 ml of the buffer, and the radioactivity was determined by liquid scintillation spectrometry, using 10 ml of Aquasol as the scintillation cocktail.

The effects of in vitro addition of DFP (10^{-9} to 10^{-4} M) on ^{3}H-GABA (15 nM) uptake was also investigated using the procedures just described.

^{3}H-GABA release from striatal slices:

The ^{3}H-GABA release studies were conducted as described earlier[23]. Slices having dimensions of approximately 0.5 X 1 X 1 mm were preincubated at 37°C for 10 min in ml of Ca^{++}-free Krebs bicarbonate-EGTA (referred hereafter as Krebs medium) medium consisting of: 124 mM NaCl, 5 mM KCl, 1.3 mM MgCl, 1.2 mM $KHPo_4$, 26 mM $NaHCO_3$, 1 mM EGTA, 10 mM glucose, 20 µM AOAA, adjusted to pH 7.4 by bubbling with 95% 0-5% CO (carbogen). slices were transferred to the Krebs medium containing 0.1 µM ^{3}H-GABA (specific activity 33.3 Ci/mmol) and incubated at 37°C for 20 min. The slices were washed twice with ml of the Krebs medium every 5 min for 20 min to observe spontaneous release. To observe the Ca^{++}-dependent K^{+}-stimulated GABA release, the slices were transferred to Ca^{++}-K^{+} Krebs medium every 5 min for 25 min. The Ca^{++}-K^{+} Krebs medium contained: 4 mM CaCl, 60 mM KCl, 64 mM NaCl, 1.3 mM MgCl, 1.2 mM $KHPO_4$, 26 mM $NaHCO_3$, 10 mM glucose and 10 µM AOAA. All incubations were carried out at 37°C under carbogen atmosphere in a Dubnoff metabolic shaker. After appropriate incubation, an aliquot of the medium from each time point was transferred to scintillation vials and counted for radioactivity. The % of release of ^{3}H-GABA was calculated as 100 x (dpm of released GABA)/(dpm of accumulated GABA in the slices).

Determination of protein concentration

The protein content of the homogenates used in the present study was determined by the method of Lowry et al[19].

Statistics:

Data from the ^{3}H-GABA uptake studies were evaluated according to Lineweaver and Burk[16]. Linear regression analyses were used to calculate the affinity constant (K_m) and uptake velocity (V_{max}). When applicable data were analyzed for significance by Student's t test: P value < 0.05 between two means was considered significant.

Results:

Effect of DFP on general behavior of animals:

Acute administration of DFP, 1 or mg/kg, s.c., resulted in prominent parasympathetic overactivity which consisted of salivation, lacrimation, urination, excessive bronchial secretions, sweating, defecation and diarrhea. Skeletal muscle twitching, fasciculations and tremors were also observed. Animals in the sub-acute treatment group continued to exhibit the symptoms for 4 to 7 days; thereafter, the symptoms regressed slowly, and by the 14th day the animals no longer showed any symptoms.

Effect of DFP on AChE and CAT activities:

As previously reported[36] the control AChE activity was 186 ± 9 nmoles/min/mg protein. DFP inhibited AChE activity in a dose-related fashion. Six hr after 1 and mg/kg DFP, the inhibition of AChE activities was 53 and 84% of the control, respectively. Even at 24 hr after the administration of a single dose of DFP, the inhibition remained around 50% of the control activity. A 30% inhibition could be observed 14 days after a single dose of

DFP. After sub-acute administration, the inhibition of AChE activity was maintained at about 70% of the control. The control CAT activity was 113 ± 8.6 nmoles/hr/mg protein. The ChAT activity remained unaltered by all the treatments.

Effect of DFP on GABA and glutamate levels:

Twenty-four hr after acute treatment with 2 mg/kg of DFP, levels of both GABA and its precursor, glutamate, were significantly elevated. None of the other acute treatments caused any significant changes (Table I). In animals continuously treated with DFP for 4 days GABA and glutamate levels were also increased; continuous treatment for 14 days failed to change the levels significantly.

Effect of DFP on ^{3}H-GABA uptake:

Twenty-four hr after a single injection of DFP 2 mg/kg, there was a significant decrease in the uptake velocity (V_{max}) of GABA. A similar effect was observed after continuous administration for 4 or 14 days. None of the treatments affected the affinity constant (K_m) (Table II).

Effect of DFP on ^{3}H-GABA release:

Six hr after doses of 1 or mg/kg of DFP, there was a significant decrease in the basal release of GABA; however there was no alteration in basal release 24 hr after the treatments. Continuous administration for 4 or 14 days also decreased basal GABA release. Neither acute nor chronic treatments altered the K^{+}-induced Ca^{++}-dependent GABA release (Table III).

Effect of DFP on GABA related enzymes:

None of the treatment schedules altered GAD and GABA-T activities (Table IV).

Discussion:

The literature dealing with the involvement of GABA in the effects produced by organophosphates is limited to a few which have examined the convulsive action of organophosphates[14,17,20,21,29]. A systematic examination of acute and chronic effects of organophosphates on GABA synaptic function is lacking. Recently we reported[36] that postsynaptic GABA receptor density was increased after acute and sub-acute treatment with DFP. The present study examined the effects of DFP on various steps involved in GABA synaptic neurotransmission.

In order to assess the state of the cholinergic system, we monitored the activities of AChE and CAT in the rat striatum. The present results confirm a previous report[36] that AChE activity was inhibited in a dose-dependent manner by acute DFP administration; continuous administration of DFP led to behavioral tolerance despite the lack of reversal of AChE inhibition. This is consistent with earlier reports[31-33,36] by other investigators. The present study also showed that CAT, the enzyme involved in the synthesis of ACh was not affected by acute or sub-acute treatments. Russell[33] has pointed out that CAT appears unlikely to be a rate-limiting step in ACh synthesis because of its presence in large excess of maximum turnover rates of

ACh, and because its inhibition has little effect on ACh levels. It is not clear whether the lack of effect of DFP on CAT activity is related to these factors.

The present study revealed that DFP (a) did not influence the metabolic enzymes of GABA, GAD and GABA-T, (b) increased the GABA and glutamate levels, (c) decreased the uptake of GABA and (d) decreased the spontaneous release of GABA. Our previous report showed that postsynaptic GABA receptors were increased by DFP treatment. These data, taken together indicate that organophosphate-induced alterations in GABA synaptic function may, at least partly, influence the overall outcome of organophosphate poisoning. Apparently, DFP effects pre- and post-synaptic mechanisms. It is well-recognized that GABA is a major inhibitory transmitter in the mammalian CNS. Since cholinergic function is greatly increased due to inhibition of AChE by DFP, a possible compensatory increase in GABAergic activity may be expected. The increase in GABA levels is probably due to the decrease in spontaneous release. In order to compensate for this effect, the uptake process may be inhibited so that availability of the transmitter to the postsynaptic sites is not grossly impaired. As mentioned earlier, anticonvulsants which are believed to act primarily via GABAergic mechanisms block organophosphate-induced convulsions[17,20].

Lundy et al[20] showed that small amounts of benzodiazepines [which are believed to act through enhancing GABAergic trans-

mission[25]] totally inhibited organophosphate-induced convulsions, whereas, even in high doses, the antimuscarinic compound atropine, had no effect. These authors further demonstrated that aminooxyacetic acid (AOAA) which elevates GABA levels by inhibiting GABA-T, also inhibited the convulsions. Certain organophosphates which do not inhibit AChE, but share the convulsive property, are believed to act through alterations in GABA function. The evidence therefore, appears to indicate that an overall GABA receptor activation may play a part in the acute effects of organophosphates.

As mentioned earlier, the overt parasympathetic activity seen after acute exposure is no longer seen after continuous administration though there was no reduction in the AChE inhibition. The next question is: how are GABA synaptic changes related to the phenomenon of DFP-induced behavioral tolerance? In general this phenomenon is attributed to neuronal adaptability which is seen with a variety of drugs such as narcotics, barbiturates etc. However, the neurochemical events underlying this phenomenon are largely unknown. It should be noted, however, that the reduced muscarinic receptor density found to occur during tolerance, is one of the mechanisms suggested for the tolerance development[2,5,9,35,36]. The trend of GABA synaptic changes seen after acute administration of DFP, was maintained after chronic treatment. It appears therefore, that the acute effect on the GABA system might be due to a compensatory effect

to counteract immediate cholinergic overactivity; the effect also appears to be maintained on the GABA system irrespective of the behavioral tolerance to continuous DFP treatment.

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Table I

Effect of acute and sub-acute administration of DFP on GABA and glutamate levels in rat striatum.

DFP Treatment	% change of control	
	GABA	Glutamate
Acute		
1 mg/kg (single dose)		
After 6 hr	+11	-3
24	+12	+3
mg/kg (single dose)		
After 6 hr	+1	+10
24 hr	+28*	+37**
7 days	-8	+1
14 days	-3	+
Sub-acute		
1 mg/kg/day x 4	+18*	+17*
1 mg/kg/day x 14	+1	+14

The control levels of GABA and glutamate, were 2.13 ± 0.12 and 12.63 ± 1.32 μmole/g tissue respectively. + and - sign represent increase and decrease from the control respectively. Each group consisted of 4 samples. *$p < 0.05$, **$p < 0.01$ compared to control (computed on the original data before normalization into % change of control).

Table II

Effect of acute and chronic administration of DFP on ^{3}H-GABA uptake of the rat striatal homogenate.

DFP Treatment	^{3}H-GABA Uptake	
	K_m (nM)	V_{max} (pmole/g protein)
Acute		
Control	155 ± 7.9	10.4 ± 1.0
1 mg/kg (single dose)		
After 6 hr	149 ± 7.3	9.4 ± 0.6
24 hr	161 ± 7.4	8.7 ± 0.4
mg/kg (single dose)		
After 6 hr	144 ± 8.3	8.6 ± 0.5
24 hr	143 ± 5.6	6.1 ± 0.4*
Chronic		
Control	125 ± 8.2	10.3 ± 0.4
1 mg/kg/day x 4	109 ± 11.0	6.62 ± 0.3*
1 mg/kg/day x 14 days	113 ± 10.0	6.73 ± 0.2*

Data represent mean ± S.E. of three determinations. ** $P < 0.05$ compared to the respective control.

Table III

Effect of acute and chronic administration of DFP on ^{3}H-GABA release from rat striatal slices.

Treatment	% ^{3}H-GABA release							
	Basal release				Ca^{++} dependent High K^{+} induced release			
	Time (min)							
	5	10	15	20	25	30	35	40
Acute								
Control	7.9 ± 0.8	6.4 ± 1.0	5.1 ± 0.8	4.0 ± 0.4	17.5 ± 1.8	12.1 ± 1.0	9.1 ± 0.3	7.4 ± 0.4
1 mg/kg (single dose)								
After 6 hr	6.6 ± 0.5*	3.8 ± 0.3*	3.3 ± 0.2*	2.8 ± 0.1*	15.8 ± 0.9	10.5 ± 0.6	8.6 ± 0.5	7.1 ± 0.5
24 hr	7.9 ± 0.9	6.4 ± 0.6	4.8 ± 0.5	4.3 ± 0.4	18.6 ± 1.0	10.3 ± 1.1	8.2 ± 0.9	6.3 ± 0.4
mg/kg (single dose)								
After 6 hr	8.7 ± 1.1	4.4 ± 0.8	3.3 ± 0.3*	2.9 ± 0.2*	15.3 ± 1.4	9.9 ± 0.5	8.8 ± 0.4	7.1 ± 0.6
24 hr	10.9 ± 1.1	8.0 ± 1.2	5.0 ± 0.3	4.2 ± 0.3	15.1 ± 0.8	10.1 ± 0.4	8.4 ± 0.4	6.4 ± 0.5
Chronic								
Control	7.7 ± 0.4	6.2 ± 0.7	4.0 ± 0.3	4.1 ± 0.7	14.0 ± 1.0	10.5 ± 0.8	6.3 ± 0.3	6.1 ± 0.2
1 mg/kg/day x 4	5.8 ± 1.5*	3.4 ± 0.6*	2.7 ± 0.4*	2.2 ± 0.2	10.4 ± 1.5	8.9 ± 0.4	7.0 ± 0.3	6.4 ± 0.2
1 mg/kg/day x 14	5.7 ± 0.5**	3.3 ± 0.3**	2.6 ± 0.4*	2.4 ± 0.4*	12.9 ± 0.6	10.6 ± 0.6	7.6 ± 0.1	6.2 ± 0.3

Data represent mean ± S.E. of 5 samples. *P < 0.05; **P < 0.01 compared to the respective control.

Table IV

Effect of acute and sub-acute administration of DFP on GABA-related enzyme activities of rat striatum

DFP Treatment	GAD (nmole/hr/mg)	GABA-T (nmole/hr/mg)
Acute		
Control	31.2 ± 0.32	196 ± 23
1 mg/kg (single dose)		
After 6 hr	38.2 ± 0.33	169 ± 21
24 hr	29.8 ± 0.30	177 ± 19
mg/kg (singie dose)		
After 6 hr	30.4 ± 0.22	166 ± 25
24 hr	27.2 ± 0.48	181 ± 11
Sub-acute		
Control	27.4 ± 1.20	230 ± 17
1 mg/kg/day x 4	31.2 ± 1.44	236 ± 12
1 mg/kg/day x 14	27.8 ± 2.41	223 ± 10

Data represent mean ± S.E. Four to 5 samples were used in each group.

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ADB086796	ADB087014	ADB094577	ADB077216
ADB089639	ADB094564	ADB097240	ADB086978
ADB087493	ADB068920	ADB108614	ADB092559
ADB086227	ADB086515	ADB111422	ADB094345
ADB096009	**ADB091643**	ADB091748	ADB113188
ADB097567	ADB097568	ADB081493	

2. The point of contact for this request is Ms. Virginia Miller, DSN 343-7327.

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(23)

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